

# Auxin Levels Regulate the Expression of a Wound-Inducible Proteinase Inhibitor II-Chloramphenicol Acetyl Transferase Gene Fusion *in Vitro* and *in Vivo*<sup>1</sup>

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## ABSTRACT

Proteinase inhibitor genes are expressed in solanaceous and leguminous plants following wounding of the foliage by mechanical methods. Previous studies have shown that a cloned proteinase inhibitor II-chloramphenicol acetyl transferase (*pin2*-CAT) chimeric gene is regulated in a wound-inducible manner in transgenic plants. In this study, we analyzed transgenic plant tissues for expression of the *pin2*-CAT gene in response to various plant hormones. We found that CAT activity was induced in tobacco (*Nicotiana tabacum*) callus incubated in the absence of any plant growth regulators. Addition of growth regulators to the medium thus permitted us to measure the effects of these substances on the activity of the *pin2*-CAT gene construction. Cytokinin (BAP) and ethylene (ethophon) even at low concentrations stimulated the expression of CAT activity by 25 to 50%. Abscisic acid at concentrations up to  $4.4 \times 10^{-5}$  molar had no effect upon CAT activity, but increasing auxin (naphthalene acetic acid) levels completely inhibited the synthesis of CAT protein. Gibberellic acid had little effect except at very high concentration ( $2.9 \times 10^{-5}$  molar). The kinetics of activation of the *pin2*-CAT gene were quite long (5 to 7 days) when unwounded calli were plated on media lacking auxin. This effect was documented for calli derived from several transformed plants, containing the full, chimeric *pin2*-CAT (pRT45) gene. In addition, calli from tissues transformed with wild-type vectors or from several plants transformed with pRT50 (a noninducible derivative of pRT45) were not induced by plating on media lacking auxin. Other naturally occurring and synthetic auxins had similar effects to naphthalene acetic acid in inhibiting the induction of the chimeric gene fusion. Finally, leaf discs from transformed plants were induced by incubation in MS liquid medium in the presence and absence of naphthalene acetic acid. NAA was also effective in down regulating the chimeric gene in whole plant tissues.

which encode quantitative insect resistance factors, are induced from a quiescent to an active state by wounding.

The complete mechanism by which these genes become induced is not known, but several steps in the process have been elucidated. Wounding of plant leaves releases intravacuolar glycosidases which interact specifically with adjacent cell walls to liberate low mol wt oligosaccharides (2, 6). These low mol wt oligosaccharides have been isolated and can be fed through the petiole of detached leaves to induce the accumulation of the proteinase inhibitors (24). The induction is regulated at the transcriptional level and the levels of mRNA approach 0.7% of total poly(A<sup>+</sup>) mRNA following multiple wounds (11). The kinetics of induction for both families of proteinase inhibitors are quite long (4-6 h) following wounding before mRNAs begins to accumulate (11). This long preinduction period is unusual in inducible systems indeed even among other wound-inducible systems gene activation is quite rapid (17).

The genes which code for these proteinase inhibitors have been isolated and characterized from both tomato and potato (5, 9, 16-18, 31). The wound-inducible nature of the *pin2* genes was demonstrated independently by two separate groups (23, 31).

This study utilizes whole plants and tissues transformed with the wound-inducible *pin2*-CAT gene to examine the biochemical mechanism of wound-induction of plant defense genes.

## MATERIALS AND METHODS

Plant hormones and media components for plant tissue culture were purchased from Gibco Laboratories, Grand Island, NY; Kelco Division of Merck and Co., San Diego; or Sigma Chemical Co., St. Louis. [<sup>3</sup>C]Chloramphenicol (specific activity = 60 mCi/mmol) was obtained from New England Nuclear, Boston, MA. All other materials were of reagent grade and obtained through local sources.

### Plant Lines and Tissue Culture

The construction and description of wound-inducible transformed *Nicotiana tabacum* cv Xanthi plants, was previously

<sup>1</sup> Abbreviations: *pin2*, proteinase inhibitor II gene; CAT, chloramphenicol acetyl transferase; NAA,  $\alpha$ -naphthalene acetic acid; IAN, indole acetamide; IPA, indole propionic acid; IBA, indole butyric acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; BAP, benzylaminopurine.

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described (31). Calli were initiated from these transformed tobacco plants by plating leaf and/or stem pieces onto callus induction medium (Murashige and Skoog (21) [MS] solid agar containing 2 mg/L of NAA, 0.5 mg/L BAP, and 200 mg/L kanamycin sulfate). This procedure produced light yellow, friable, rapidly dividing calli for each of the transformed tobacco lines. Transformed calli were maintained in an illuminated incubator set to 16 h days at 26°C and 8 h nights at 18°C. The calli were expanded on the above medium or a similar medium in which IAA replaced NAA. To induce the activation of the *pin2*-CAT gene in transformed tissues, calli portions (approximately 0.5 cm in diameter) were transferred to basal medium (MS agar) containing differing concentrations of phytohormones as indicated. One tobacco cell line, NT-RT45-01, was produced by cocultivation of *N. tabacum* cv Xanthi plants with the wild type *Agrobacterium tumefaciens*, A281. This cell line has been maintained on media without hormones or on regeneration media (MS plus 0.5 BAP) for more than 2 years, yet it has never regenerated plants.

#### CAT Assays

Extracts of tobacco calli were prepared by grinding the tissue plus an equal volume of homogenization buffer (0.1 M Tris-HCl [pH 8.0], 0.5 M sucrose, 0.1% [w/v] ascorbic acid, 0.1% [w/v] cysteine HCl) in a Con-Torque homogenizer (Eberbach, Corp; Ann Arbor, MI). Following grinding, the expressed juice was twice centrifuged for 5 min at 10,000g and the clear supernatant was recovered for protein analysis by the method of Bradford (4). A quantity from each extract containing 100 µg of protein was assayed for CAT activity using [<sup>14</sup>C]chloramphenicol (100,000 cpm = 1.66 µmol) as substrate (10). Following TLC analysis, the activity of the CAT protein was visualized by exposure to x-ray films. The TLC plates were overlayed onto the exposed film and the radioactive spots corresponding to labeled chloramphenicol acetate and unreacted chloramphenicol were cut out and counted in a Packard liquid scintillation counter. Data are expressed as the percent conversion of chloramphenicol into the acetylated forms of chloramphenicol in 60 min at 37°C.

#### Plant Leaf Induction

Leaf discs were removed from the leaves of transformed plants by a cork punch and the epidermis peeled from them. These discs were induced by a method of R. Johnson and C. A. Ryan (personal communication). Leaf discs were incubated in Murashige-Skoog liquid medium containing various amounts of naphthalene acetic acid. After 18 to 24 h the leaf disc tissues were ground and CAT assays were run as described above.

#### RESULTS

##### Induction of *pin2*-CAT on Basal Medium

To induce the accumulation of CAT protein, calli prepared from plants containing the wound-inducible chimeric *pin2*-CAT gene were placed in tissue culture on basal medium.

This caused an activation of the wound-inducible gene and permitted us to begin an investigation of the hormonal effects on the regulation of this gene.

In order to begin these studies on the hormonal regulation of this *pin2*-CAT construction, it first became necessary to determine the kinetics of activation of the chimeric gene in calli transferred to basal medium. Therefore, a callus line from the transformed plant, Tr12 (31), was inoculated for 0 to 10 d on various test media. The amount of CAT activity was measured by TLC assay and was taken to represent the activity of the wound-inducible *pin2* promoter. Figure 1 shows the accumulation with time of CAT activity, expressed as percent conversion of chloramphenicol into chloramphenicol acetates.

The accumulation of CAT activity in the Tr12 calli on basal media was negligible until d 5, then over a 48-h period large amounts of CAT activity were induced. In contrast to this, when calli were plated on the maintenance medium, (basal MS medium plus 2.0 mg/L NAA, 0.5 mg/L BAP) no expression of CAT activity was apparent after 10 d. In addition, when Tr12 calli were plated on basal media for 5 d until CAT activity was just beginning to be expressed, and then they were shifted onto maintenance media, no significant CAT activity was obtained. Thus, shifting onto fresh media containing NAA and BAP reversed this induction process. This implied that the 5-d period of induction may have exhausted some callus component and this depletion leads to activation of the *pin2*-CAT gene.

#### Auxin Regulates *pin2*-CAT Gene Expression

The two components of the maintenance media that are not found in the basal medium are cytokinin (BAP) and auxin

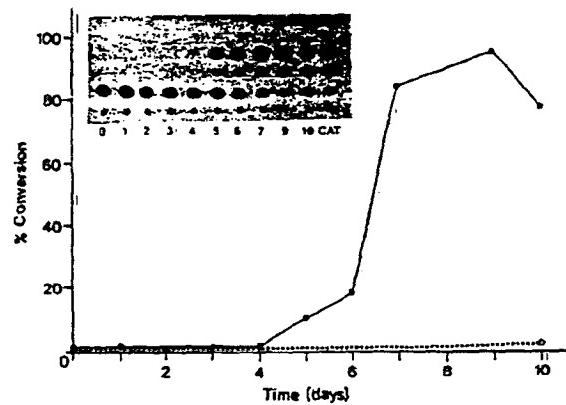


Figure 1. Time course of induction of CAT activity in transgenic Tr12 tobacco calli following transfer to hormone-free medium. The data presented are the percent conversion of chloramphenicol into chloramphenicol acetates. Calli were grown on media containing 2 mg/L NAA, 0.5 mg/L BAP, and on d 0 were transferred onto basal media lacking hormones (closed circles), or complete media containing 2 mg/L NAA, 0.5 mg/L BAP (open circles). In this experiment, three calli at each time point were pooled and homogenized together. The data presented are thus an average of the individual calli. The inset is an autoradiograph showing expression of CAT activity at the indicated days; 0 and CAT are negative and positive CAT enzyme controls.

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(NAA). Therefore, the effects of these plant hormones on the regulation of *pin2*-CAT gene expression was investigated (Fig. 2). BAP, when added at even the lowest concentration ( $8.8 \times 10^{-8}$  M), stimulated the expression of CAT activity by about 30% over that obtained with the basal medium alone. This stimulation did not significantly change with increasing concentration of BAP up to  $4.4 \times 10^{-7}$  M.

In contrast, when the auxin naphthalene acetic acid was added to the basal medium either alone or in combination with cytokinin, the expression of inducible CAT protein was drastically reduced. Thus, auxin concentrations in the media apparently regulate the *pin2*-CAT gene. The hormone concentration required to give 50% inhibition of expression was  $38 \mu\text{g/L}$  or  $2.0 \times 10^{-7}$  M NAA, which is near to physiological concentrations of IAA in tobacco leaves (28).

When the cytokinin, BAP was added to media containing various levels of NAA, the expression of *pin2*-CAT gene closely resembled the expression found with NAA alone (data not shown). We therefore concluded that auxin levels control expression of CAT activity in these tissues and that the increased activity induced by cytokinins in the complete absence of auxin may not be physiologically relevant.

This phenomenon was initially observed with callus derived from a single transformed plant, Tr12. To determine whether this was a unique phenomenon restricted to this transformant or whether this was a general characteristic of the chimeric wound-inducible *pin2*-CAT gene fusion, calli derived from other transformed plants were tested in this same manner. In Table I, calli derived from three independently transformed plants Tr12, Tr24, and Tr31, each transformed with the construction pRT45 (31), were examined for their induction

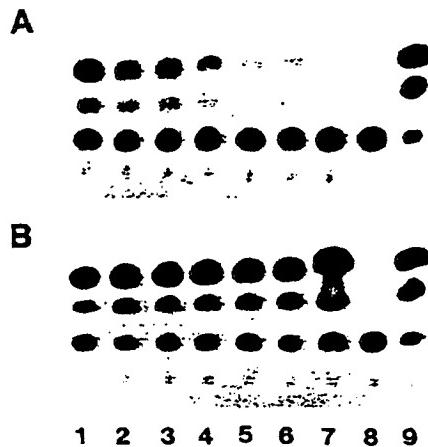
on auxin-less media. All three of these transformed lines were induced to high levels on the basal media. The levels of induction ranged from 15- to 50-fold higher than the levels of CAT protein induced in whole, wounded plants. The transformant which is most highly inducible by wounding (Tr24) is also most inducible by auxin depletion. In addition, three other transformants, Tr18, Tr19, and Tr62, were also tested. These plant lines were transformed with pRT50, a derivative of pRT45 in which the inhibitor II transcriptional terminator is replaced with the terminator from transcript 7 of *Agrobacterium tumefaciens* T-DNA (31). Plants transformed with this construction are drastically reduced in their response to wounding. Calli derived from these plants do not express CAT protein in the absence of auxin (Table I). Finally, one additional cell line was tested, construction pRT45 was transferred into *Nicotiana tabacum* cv. Xanthi using a wild-type *Agrobacterium* vector, A281. The *pin2*-CAT gene in this cell line was not inducible by plating onto auxin-free medium.

Since auxins appear to regulate the expression of this chimeric *pin2*-CAT gene fusion in transgenic tobacco tissues analogous to wounding of whole plants, we examined the regulation of this gene by other endogenous and exogenous auxins. As shown in Table II, auxin-less media permitted the activation of the wound-inducible CAT gene fusion in Tr12 calli to a high level. NAA at  $2 \text{ mg/L}$  ( $1.2 \times 10^{-5}$  M) effectively blocked the expression of CAT activity. However,  $\beta$ -NAA was ineffective in repressing the expression of the wound-inducible CAT gene activity. This correlates with previous published reports that  $\beta$ -NAA is not an active auxin (28). Endogenous auxins such as IAA, IPA, IBA, or IAN all repressed the expression of the wound-inducible CAT gene fusion but not as effectively as NAA. The synthetic auxin analogs 2,4-D and 2,4,5-T were approximately 10-fold better than the endogenous auxin, IAA, in repressing the expression of the CAT activity.

Other hormones were also tested for their effect on the expression of the *pin2*-CAT gene in transgenic callus. None of the other hormones tested were able to induce the gene when the hormones were added to maintenance medium; however, when added to the basal medium, ethylene (ethephon, 2-chloroethyl phosphonic acid) increased the level of CAT expression by 25 to 50%, even at the lowest concentrations tested ( $1.4 \times 10^{-7}$  M). The nature of this ethylene stimulation has not been further investigated, but since ethylene is produced following wounding, this may be an amplification system to super-stimulate the levels of wound-inducible gene expression. Abscisic acid (at concentrations up to  $3.8 \times 10^{-5}$  M) had no effects upon the action of the wound-inducible *pin2*-CAT gene and gibberellic acid had inhibitory effects at very high concentrations ( $2.9 \times 10^{-5}$  M).

#### Auxin Regulates *pin2*-CAT Expression in Vivo

To determine whether this auxin-dependent regulation of the chimeric *pin2*-CAT gene fusion in transgenic tobacco tissues was also operating in whole plants, we examined CAT gene activity in whole plant tissues by a leaf disc assay system. Our initial experiments with leaf discs did not respond to auxin. This was apparently due to the inability of auxin to penetrate the leaf epidermis. When dimethyl sulfoxide is



**Figure 2.** Expression of CAT activity in calli from Tr12 on media containing various hormone levels. Calli were incubated for 7 d on the various media and then processed for CAT assay as described in "Materials and Methods." A, Addition of NAA to the basal medium; B, addition of BAP to the basal medium. Lane 1, no addition; lane 2,  $0.02 \text{ mg/L}$ ; lane 3,  $0.05 \text{ mg/L}$ ; lane 4,  $0.2 \text{ mg/L}$ ; lane 5,  $0.5 \text{ mg/L}$ ; lane 6,  $2.0 \text{ mg/L}$ ; lane 7,  $10 \text{ mg/L}$ ; lane 8, negative control; lane 9, positive control containing 0.1 unit of purified *Escherichia coli* CAT enzyme. Control levels of CAT activity were 55% conversion of chloramphenicol into chloramphenicol acetates.

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Table I. Inducibility of Various Transformed Plants and Tissues

Construction	Transformant	Conversion		Inducibility	Conversion <sup>a</sup>
		Unwounded	Wounded		
		%	%		%
pRT45	Tr12	0.4 ± 0.1	0.8 ± 0.3	2.0	38.4 ± 13.0
pRT45	Tr24	0.5 ± 0.2	3.8 ± 2.8	9.6	59.9 ± 17.3
pRT45	Tr31	0.8 ± 0.4	1.6 ± 1.3	2.0	25.5 ± 12.4
pRT45	NT-RT45-01 <sup>c</sup>				1.4 ± 0.6
pRT50	Tr18	0.3 ± 0.1	0.3 ± 0.1	1.0	0.5 ± 0.1
pRT50	Tr19	0.4 ± 0.3	0.4 ± 0.2	1.0	1.1 ± 0.3
pRT50	Tr62	0.6 ± 0.5	0.6 ± 0.4	1.0	0.5 ± 0.1

<sup>a</sup> Wound-induced expression of the CAT gene in transgenic tobacco plants transformed with either pRT45 or pRT50 (see ref. 31 for details of transformation and plasmid construction). Several clonal propagants of each transformant were assayed by the previously described wound assay (31). The number of each wounded transformed plant compiled to yield these data are: Tr12, n = 5; Tr24, n = 9; Tr31, n = 6; Tr18, n = 5; Tr19, n = 7; Tr62, n = 4. The data is presented as the mean ± sd of the % conversion of [<sup>14</sup>C]chloramphenicol into [<sup>14</sup>C]chloramphenicol acetates by 100 µg of protein from an unwounded control leaf and 24-h wounded leaf. The inducibility represents the ratio of the means of % conversion in the wounded leaf to the unwounded leaf of the same plant. <sup>b</sup> Calli derived from each of the transformed plants were plated on media lacking auxin and after 7 d were assayed for CAT activity. The data presented are the means ± sd of the % conversion of [<sup>14</sup>C]chloramphenicol into [<sup>14</sup>C]chloramphenicol acetates by 100 µg of protein from the calli; n = 4. <sup>c</sup> A cell line derived from transformation of *N. tabacum* cv Xanthi with a wild type Agrobacterium, A281; n = 6.

Table II. Effect of Auxins on pin2-CAT Gene Expression

Auxin	Concentration	Conversion <sup>a</sup>	
		µg/mL	%
None			83.2 ± 3.0
α-NAA	2		4.5 ± 2.4
β-NAA	2		84.7 ± 7.2
IAA	2		9.9 ± 4.1
IPA	2		17.0 ± 10.2
IBA	2		17.6 ± 13.7
IAN	2		10.1 ± 2.0
2,4-D	0.2		29.0 ± 10.6
2,4,5-T	0.2		6.2 ± 2.6

<sup>a</sup> Conversion of [<sup>14</sup>C]chloramphenicol to [<sup>14</sup>C]chloramphenicol acetate by 100 µg of extracted plant protein. Data presented are the mean ± sd; n = 4. All auxin values except β-NAA differ significantly from control at the 99.9 confidence level.

included at 2 ppm (Fig. 3, lanes 1–3) to facilitate cell entry, the auxin does indeed down regulate pin2-CAT gene expression; however, high concentrations were required to completely turn off gene expression suggesting that this procedure may not allow full equilibration of auxin across the epidermis. When the epidermis was removed, lanes 4 to 6, lower levels of auxin (<10 µg/ml) were required to completely repress activity of the wound-inducible chimeric gene in whole plant tissues.

When leaf discs were incubated in MS media containing cytokinin alone, the pin2-CAT gene was still induced to high levels (not shown). Since cytokinin alone can maintain the viability of solanaceous leaf tissue, this suggests that viable leaf tissue is responding to the absence of auxins in these whole plant tissues.

## DISCUSSION

Transgenic tobacco callus containing a wound-inducible proteinase inhibitor II-CAT gene fusion is regulated in callus

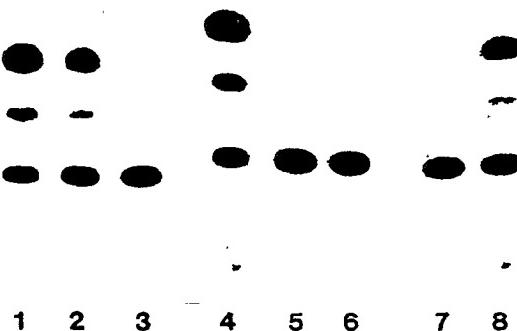


Figure 3. Expression of CAT activity in leaf discs from a Tr25 plant incubated in MS liquid medium alone or with the addition of NAA. Lanes 1 to 3 contain 2 ppm dimethyl sulfoxide to aid penetration of the epidermis by the auxin. Leaf discs in lanes 4 to 6 have the epidermis removed prior to incubation. Leaf discs were incubated for 18 h in the MS media and then processed for CAT assay as described in "Materials and Methods." CAT assays were performed with 100 µg of total leaf protein. Lanes 1 and 4, no addition; lanes 2 and 5, 10 µg/L NAA; lanes 3 and 6, 100 µg/L NAA; lanes 7 and 8 are negative and positive controls, respectively, containing either no addition or 0.1 unit of purified *E. coli*-CAT enzyme.

tissues and in leaf discs by exogenously applied auxin. Other hormones had either little or slight stimulatory effects on the expression of pin2-CAT gene in the absence of auxin. This auxin effect is specific and has been shown to function in several independently derived wound-inducible transformants. Levels of CAT expression in transgenic callus maintained on basal medium correlated to the wound-inducible expression found in whole plants. Plants which showed the highest levels of wound-inducible expression of CAT protein also showed the greatest turn-on in the absence of auxin in

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callus. The standard deviation of these auxin studies was frequently high and several factors may contribute. The size of the explant may be one of these factors as well as the nature of cut surfaces on the explant.

Lines of transgenic callus derived from plants which contain a noninducible derivative of pRT45 are not affected by auxin. Additionally, a cell line derived from transformation of tobacco with wild-type *Agrobacterium tumefaciens* vectors did not induce the *pin2-CAT* gene by plating onto hormone free media. Such transformed calli frequently contain independently cotransformed T-DNA sequences, along with the gene of interest (7). This cell line has been maintained on media without hormones or on regeneration media (MS plus 0.5 BAP) for more than 2 years, yet it has never regenerated plants. Thus, this cell line NT-RT45-01 likely contains the wild-type T-DNA locus as well as the pRT45 locus. The T-DNA locus contains a well characterized pathway for the production of IAA in the plant tissues (30). This pathway aids in maintaining the callus in the undifferentiated state (27). Presumably, since these calli produce their own auxin, this cell line, NT-RT45-01, is not induced by plating these calli onto the basal media lacking auxin. We did not, however, determine the endogenous levels of auxin in the NT-RT45-01 calli.

Whether changes in auxin homeostasis permits other plant defense genes to be induced is not known; however, exogenously applied auxins have been shown to repress invertase in Jerusalem artichoke tuber slices (8), peroxidases in young peas (22), and another potato proteinase inhibitor (25). In addition, exogenously applied auxins have been reported to induce susceptibility of maize to *Helminthosporium* leaf spot (15) and of tobacco to tobacco mosaic virus (29). Thus, auxin fluxes may also play a role in the regulation of several plant defense genes.

Further, since increasing auxin down-regulates this plant defense gene, it is possible that this may play an important role in plant pathogenesis by microorganisms. Many plant pathogens endogenously produce auxin (20) and this auxin is thought to be a virulence factor for some pathogens (27). Production of sufficient amounts of auxin in the infected tissue could effectively block the expression of this or other auxin-repressible plant defense genes.

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